CATHELCO LTD
BALLAST WATER MANAGEMENT SYSTEM

PART 4 ANNEX 2a

MEA Test Protocol and QAPP

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MARINE ECO ANALYTICS

TEST PROTOCOL FOR THE SHIPBOARD VERIFICATION OF THE BALLAST WATER TREATMENT TECHNOLOGY OF

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Including Quality Assurance Project Plan (QAPP)

Content

Water and sediment of ships should be free of allien invasive organisms

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CHAPTER 1

Introduction

This protocol is based on guideline G8 (Anon, 2008) of the IMO.

1.1 Purpose of ship board verification testing program

The purpose of the ship board testing program of a ballast water treatment system (BWTS) is to determine the overall performance and in particular the efficacy to remove or kill organisms under normal operational conditions. The ballast water treatment procedures should be conducted as part of the standard procedures of the ship and carried out by the ship's crew according the operational and maintenance manual of the manufacturer.

The systems performance should be verified under normal operational procedures in water quality conditions which do represent natural conditions in terms of abiotic and biotic characteristics. In a separate test program (land-based testing) the BWTS should be tested under far more extreme conditions to guarantee a broad range of operational conditions.

CHAPTER 2

Description of the ballast water treatment system and the ship

2.1 Technical specifications of the ballast water treatment system (BWTS)

Cathelco has developed a 100% chemical free BWTS. It is based on the combination of filtration + UV treatment. There are no active substances needed for the treatment of the ballast water, or for the cleaning of the UV system. The system was designed with emphasis on retrofitting (i.e. installation of the different components as required by different engine room designs). However, skid-mounted systems will also be available, which are optimized for foot-print.

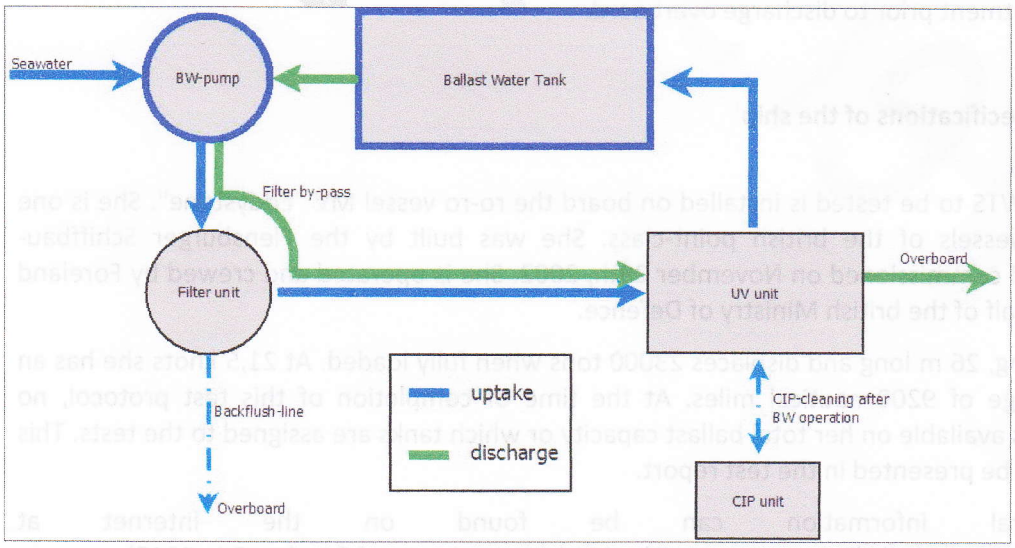

Figure 1: Process plan of Cathelco's BWTS. Water flows: blue lines: at intake; green lines at discharge

Cathelco's BWTS is a modular ballast water management system. The system is installed in bypass to the main ballast line and provides a safe, flexible and economical process for the treatment of ballast water and eradication of aquatic invasive species. On the test ship, the BWTS is installed in a void space in the engine room.

Treatment of ballast water is achieved through a simple and efficient two-step process:

Filtration

The ballast water passes through an automatic back flushing filter capable of removing particulates, and organisms (zooplankton and phytoplankton) using a 40 µm super mesh screen. The screen is arranged in cone shaped filter candles, where the number of filter candles varies with the treatment related capacity (TRC) of the BWTS. The shipboard G8-tests will to be performed at 340 m3/h flow rate, with a 400m²/h BWTS installed.

The automatic cleaning cycle of the filter is activated by an increased pressure drop across the filter. The filter candles are cleaned one after the other without interruption of the filtration process. The frequency of the filter back flushes depends on the quality of the water (e.g. the TSS content). The concentrate is discharged over board. This ensures that the screen is kept clean and the filtration process maintained at maximum efficiency at all times.

UV disinfection

The filtered ballast water is directed into a manifold that is holding two UV reactors, with a hydraulic capacity of 200m³/h, each. In each of the UV reactors, a cross flow arrangement with two medium pressure ultraviolet lamps delivers the high intensity irradiation. The UV light intensity is continuously monitored individually for each of the UV lamps inside of the UV reactors. The UV intensity is maintained above pre-set values to ensure delivery of the required dose during the entire system operation.

The ultraviolet lamps are housed within quartz sleeves and an automatic mechanical cleaning system minimizes bio-fouling and controls the accumulation of deposits on the UV lamp sleeves.

At discharge, the automatic backwash filter is by-passed and the ballast water is pumped from the tanks through the UV disinfection chamber only. Thus the ballast water is subjected to UV disinfection treatment prior to discharge overboard.

2.2 Technical specifications of the ship

The Cathelco BWTS to be tested is installed on board the ro-ro vessel MV "Eddystone". She is one of six sea-lift vessels of the british point-class. She was built by the Flensburger Schiffbau-Gesellschaft and commissioned on November 28th, 2002. She is operated and crewed by Foreland Shipping on behalf of the british Ministry of Defence.

She is 193 m long, 26 m long and displaces 23000 tons when fully loaded. At 21,5 knots she has an operational range of 9200 nautical miles. At the time of completion of this test protocol, no information was available on her total ballast capacity or which tanks are assigned to the tests. This information will be presented in the test report.

More general information can be found on the internet at http://en.wikipedia.org/wiki/Point_class_sealift_ship (site version as of October 8th, 2012).

CHAPTER 3

Parameters to be measured

The challenge conditions for ship board testing are specified into two groups of factors; general water quality conditions (abiotic factors) and numbers of living (viable) organisms as specified into different size classes.

3.1 Abiotic parameters

The guideline, Part 2, Section 2.2.2.9 requires the following abiotic parameters to be measured:

- Salinity
- Temperature
- Total Suspended Solids (TSS)
- Particulate Organic Carbon (POC)

3.2 Biological parameters

The following biological parameters will be tested if they comply with the D2-Standard of the Ballast Water Convention (IMO, 2004).

1. Viable organisms with minimum dimension equal to or larger than 50 microns
2. Viable organisms with minimum dimension smaller than 50 microns
3. Indicator microbes belonging to the group of E. coli and enterococcus

Ad 2, the size class of organisms smaller than 50 micron, will also include the organisms smaller than 10 microns and therefore covers the whole range of biological relevant size classes. The group of organisms smaller than 10 micron does include some species that can form harmful algae blooms and are therefore of particular interest in the scope of ballast water treatment. This is a deliberate deviation from the guidelines.

3.2.1 Intake and discharge requirements

The guideline, Part 2, Section 2.2.2.5 requires an uptake of organisms exceeding ten times the discharge standard. This translates into more than 100 organisms per cubic meter for the size class larger than 50. Equally more than 100 organisms per mL are required for the size class 10 – 50. Bacteria and especially the indicator microbes (human pathogens) are not specified here. Based on the relevant section for land-based test, it is assumed that valid test cycles can be done on board without human pathogens.

Upon discharge the treated water has to fulfil the requirements of the D2-Standard. The control water has to exceed the requirements upon discharge in order for the test run to be valid.

CHAPTER 4

Methodology

4.1 Sampling methodology

4.1.1 Sampling locations

Sampling will be done at intake and discharge at appropriate locations. In practice this will be at intake prior to ballast water tank and at discharge immediately after the BWTS.

4.1.2 Sample collection

All samples will be taken via a sample hose connected to an iso-kinetic sampling point in the ships ballast line. After sampling the water that is not needed will be pumped back into the main ballast line and discharged normally. If this is technically not possible, a suitable solution will be discussed with the chief engineer during the site visit and during the first (trial-)test run.

Sampling can be continuous or discrete depending on the parameter to be measured but in all cases cover the whole discharge period. In addition to water samples which are treated by the ballast water treatment system water also water from a control tank, filled parallel at intake and discharge, should be taken. All samples are taken in triplicate covering the whole intake and discharge process. Samples should be taken with minimal disturbance and additional stress to the organisms.

The sampling scheme described below for treated water differs from the guideline. There are two main reasons for this approach. Firstly the sampling strategy described in the guideline, Part 2, Section 2.2.2.6.2.1 is based on the assumption that subsamples from the sample are analysed. We will analyse the whole volume sampled. For analysis which involve subsampling (e.g. flow-cytometry) nine samples as described in the guideline will be taken. Secondly, by following the sampling strategy as described below a better comparability of results with the results from the land-based testing is given. This is due to following the same sampling strategy for ship-board and land-based testing.

4.1.3 Abiotic parameters

Salinity and temperature will be measured at different intervals in discrete samples collected in a bucket (approx. 10 L) covering the whole discharge period.

Sub-samples for TSS and POC are taken and filtered immediately after sampling (pre-weighted GF/C). Depending on the particle load this volume will be between 200 and 1000 ml per filter. Water in the bucket will be mixed prior to taking a sub-sample, since particles tend to sediment rapidly when standing still. After filtration the filters will be put back into a coded petri-dish and stored in a -20 °C freezer.

4.1.4 Biological parameters

a) Plankton larger than or equal to 50 micrometer

Control samples

Control samples will be taken by filling 20 litres into buckets and subsequently filtering this water over a 50 micron diagonal mesh (= 35 – 37 µm square). All organisms and material retained on the filter mesh will then be flushed into a clean sample bottle. Subsequent processing will be the same as for the treated samples.

Treated samples

Samples will be taken using a Hydrobios-plankton net with 50 micron diagonal mesh (= 35 – 37 µm square). The net will be suspended in either an International Bulk Container (IBC) of one cubic meter or, where the available space does not allow this, in so-called big bags whose volume will be determined and marked prior to deployment. The purpose of the containers is purely to volumetrically determine the sample size. However on board ship a portion of the water from the containers may be taken to prepare the organism free water necessary for sample processing. This will be done via a filtration cascade with an 0,2 µm filter as final step.

Samples will be taken over the whole pumping period. Individual samples should ideally be one cubic meter each, but practicality on board the vessel might dictate smaller volumes per sample. This will be determined in a technical test run prior to certification testing.

The content of the net will be flushed with organism free water into a clean sample bottle. The sample will then be transferred into a beaker and Neutral Red stain will be added.

b) Plankton smaller than 50 micrometer

Samples will be taken separately for all protists (phyto- and microzooplankton). For the latter a pre-weighted bottle with 4 ml of Lugol's-solution will be filled with one litre of water directly from the sampling hose per sample. These bottles will then be stored in the dark for microscopic analysis on land.

For phytoplankton one litre of water will be taken per sample directly from the sampling hose. This sample will be divided in various subsamples to be analysed immediately on board or at a later stage in the laboratory. One subset will be stored cool and dark (refrigerator or cooling box) for later flow-cytometric analysis on land. Another part will be directly measured on board to determine the presence and viability of the phytoplankton as a bulk parameter.

c) Bacteria (indicator microbes)

Subsamples for the indicator microbes (*E. coli* and enterococci) will be taken in special bottles provided by the test laboratory and stored at a low temperature prior to delivering them to the test laboratory. Since a too long holding time before analysis may affect the outcome of the human pathogens test for each ship board test the validity of testing for the presence of human pathogens will be determined. The outcome of the test may be affected in two different ways during a prolonged holding period. Firstly there may be on-going mortality of the human pathogens. Secondly, storage on board is not comparable with clean laboratory conditions, so samples may become contaminated, thereby introducing human pathogens.

d) Bacteria (total)

Subsamples for total bacteria numbers will be taken and preserved with glutaraldehyde or formaline, depending on availability. Final concentration for both fixatives will be 2%. Samples will be stored at -20 °C until transport. Transport will be in a cooling-box with cooling elements. Analysis by flow cytometry will be performed at the earliest possibility after transport.

4.2 Sample processing

4.2.1 Abiotic parameters (SOP 306 and SOP 309)

Salinity and temperature will be measured using a standard multi-probe (Palintest Macro 900-system).

For TSS the filters are dried at 60°C overnight and then weighed again. The concentration of TSS per litre can then easily be calculated from the sample volume and the weight difference of the filter before and after. TSS is expressed as mg per L.

For POC the filter is combusted at 550°C, allowed to cool and weighted again. The POC is calculated from the weight decrease between this measurement and the TSS weight. POC is expressed as mg C per L.

4.2.2 Biological parameters

a) Plankton larger than 50 micrometer (SOP 320)

Control and treated samples

The content of the sample bottles will be transferred into a clean beaker and Neutral Red stain will be added. After a minimum staining period of 2 hours the sample will be analysed using a dissection microscope (a Leica MX 5 -or comparable- with cold light source is used). Samples will be analysed at 20x magnification as a standard for counting, increasing up to 50x for live-dead-separation or species determination if required. The dissection microscope will be placed on a vibration absorbing rack to minimize adverse effects on sample analysis (e.g. engine vibration). Samples for this size-fraction will be always analysed entirely without sub-sampling.

b) Plankton smaller than 50 micrometer

Processing of samples for inverted microscopy (SOP 319)

Samples preserved with Lugols solution are analysed back on land. After arrival in the lab the sample bottles are put in a dark, vibration free cupboard for at least 24 hours. In this period all organic material will sediment due to the Iodium incorporated from the Lugol. Sample bottles are then weighed again and the watery phase is sucked out with a glass-pipett. The remaining concentrate is weighed again and subsequently stored in a dark bottle until further analysis. Analysis is carried out with an inverted microscope (method modified from Utermöhl 1958). A known volume of the concentrate is pipetted into a sedimentation chamber. Remaining Lugol is neutralized with sodiumbisulfate. Then the sub-sample is stained with Bengal Rose to stain organic material. Live-dead-separation in these samples is mainly based on the structural integrity of organisms. This method can be applied for both zoo- and phytoplankton.

Processing of samples for flow cytometry and PAM (SOP 317 and SOP 318)

One litre of water will be taken per sample directly from the sampling hose. This sample will be divided. Part will be stored cool and dark (refrigerator or cooling box) for later flow-cytometric measurement on land. Another part will be directly measured on board using a WALZ water-PAM to get a qualitative bulk measurement on phytoplankton vitality in the samples (Schreiber et al, 1993).

Flow cytometry:

Three replicate samples of 3 mL each will be taken from both control and treated water. They will be pipetted in an ultra-clean sampling tube and put in the carousel of a bench top flow cytometer (Beckman Coulter XL-MCL). This flow cytometer is certified by the FDA for medical laboratory use and as such maintained by the manufacturer in their service scheme. All procedure and handling are conducted according the standard procedures as described in Shapiro (Shapiro 2003). As a light source a 15 mW Argon laser is used (488 nm excitation wavelength). Forward and side scatter is detected of each particle as well as the fluorescent emission in the yellow/green (525 ± 20 BP filter), orange (575 ± 20 nm) and red wavelength band (>645 nm, for details see Veldhuis & Kraay 2000). Samples will be counted using a standard protocol covering the particles in the size range of ca. 2 to 50 µm. Total analysis time will be equal to an exact sampling volume of 1 ml (for details on volume calibration see SOP). Of all particles present in 1 ml of sample, cell size and presence or absence of chlorophyll a will be measured. Absolute numbers, cell sizes and chlorophyll a content of the particles will be analyzed using the software package FCS Express V3 (DeNovo version 3).

PAM fluorometry:

The photochemical efficiency of photosystem II (an indicator of the 'health' condition of the cell) of phytoplankton can be addressed using a Pulse-Amplitude Modulated fluorometer (PAM-fluorometry; Schreiber et al 1993). This simple parameter gives a qualitative indication of the photosynthetic activity of the phytoplankton community. For this 3 ml of unfiltered sample water (control and treated, each in triplicate) are filled into a glass cuvette and analysed using the Pulse-Amplitude Modulated fluorometer (automated measurement). In case an increase of the photosynthetic activity of the whole phytoplankton community is observed samples will be filtered (10 µm sieves) to determine the exact size class of the viable phytoplankton fraction.

c) Bacteria

Total bacteria (SOP 316)

The classical method for counting bacteria in many applications is based on plating on selective media. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol & Giorgio 2000). As a result total bacteria will be determined by flow cytometry, using DNA-specific stains to get a more accurate bacteria number. In addition samples are taken at discharge for specific human pathogens and heterotrophic bacteria using a plate method.

Upon analysis the sample is allowed to thaw completely. A subsample of 100 µL is taken, diluted with a TE-buffer, and the nucleic acid dye PicoGreen (Invitrogen) was added. Within 5 to 15 minutes after the addition of the stain the sample is analyzed using a flow cytometer (cf. Gasol & Giorgio 2000; Veldhuis et al. 1997). A known bacterial standard is used for calibration and counting.

The dye PicoGreen is a green nucleic acid specific dye that only stains dsDNA, with little or no cross-over for ssDNA and RNA (Veldhuis et al, 1997). This makes the staining with PicoGreen method ideal to for staining of DNA and therefore to determine bacterial abundance.

Human pathogens (SOP 311)

The samples for microbiological analysis are taken in special bottles of 300 or 600 mL and send to a special, contracted laboratory for further analysis. For these tests this will be Vitens b.v. in the Netherlands. Final measurements are conducted according to NEN/ISO standards. However, as acknowledged in guideline G8, transport and storage times involved in shipboard testing might affect these samples.

Escherichia coli:

Analysis for *Escherichia coli* is carried out according to ISO 9308-3 for the analysis of surface waters.

Enterococcus group:

Analysis for this group is carried out according NEN/ISO 7899-2.

CHAPTER 5

Quality Assurance/Quality Control (QA/QC)

5.1 Project Management

In 2012 a quality management system meeting the principles of ISO 9001 will be implemented. MEA-nl assures that all analysis done at external labs will be done conform the same quality requirements.

For the shipboard testing of the Cathelco BWTS, Dipl.-Biol. F. Fuhr is assigned as the responsible project manager on behalf of MEA-nl.

5.2 Measurement and Data Acquisition

Quality of measurements and data acquisition is guaranteed by having a standard operating procedure (SOP) for every measurement needed to verify a BWT system. SOPs are part of our Quality Management System, assuring that measurements are done in the same way, with the same equipment, independent of which trained employee is performing the measurement. Methods are all based on well-known scientific procedures. Data are therefore always of the same quality. All tests and measurements needed to verify a BWTS are done by experienced personnel. Personnel working from the start at MEA-nl is expected to have enough experience on methods they are responsible for. New personnel will be trained for every method they will perform on a base of: read-see-act (under supervision).

Test results will be recorded at forms attached in SOPs or in note books specified in the SOP. This assures that all data needed will be reported synchronously with the data collection. The verification strategy will be discussed in the next paragraph.

5.3 Verification of Test Data

Raw data generated during testing are important for the verification of the BWTS. Therefore these data will be recorded electronically and are stored on a regular basis. Electronic entry of raw data is the responsibility of the analyst/ scientist collecting the raw data and will be completed as soon as practically. Electronic data are checked against the raw data by someone else at MEA-nl to assure the quality. Electronic data will be stored for at least seven years. Further details of data management can be found in BP-202 *'Data and Documentation management'*.

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